Expression Pattern of PRDM Family Using Tissue Clearing Technique in Mouse Micro-embryo

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감사의 글

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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The proteins member of the PRDM family are known to have a potential role in tumor suppression, as well as in other diseases. Thus, elucidating the expression patterns of these PRDM proteins by high resolution analysis is necessary to future determine to characterize their biological role.

Recent development in tissue clearing methods such as CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/*In situ* hybridization-compatible Tissue hYdrogel) has allowed for three-dimensional analyses of biological structures as a whole, intact tissue, providing greater understanding of spatial relationships and biological circuits. Nonetheless, studies have reported issues when it comes to maintain structural integrity and preventing tissue disintegration, discouraging the application of these techniques with fragile tissues such as developing embryos.

Here, we present an optimized passive clearing technique, mPACT-A (modified PACT-Acrylamide), which improves tissue rigidity without the detriment to

optical transparency. The mPACT-A protocol is specifically optimized for handling mouse embryos, which are small and fragile and get easily dismantled when processed with established tissue clearing methods.

We demonstrated the feasibility of this technique by investigating the expression of relatively understudied PRDM proteins, PRDM7, 8, 12 and 13, in intact cleared mouse embryos at different stages of development. We observed strong PRDM7, 8, 12 and 13 expression in the developing mouse nervous system in various development stages of the mouse embryos. These results suggest potential roles for the PRDM proteins in neural development, that should be tested in future functional studies.

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I. INTRODUCTION

PRDI-BF1 and RIZ homology domain-containing (PRDM) proteins are a
family whose members are defined by a conserved N-terminal PR domain and a
variable number of zinc finger repeats in their C-terminus[1-5]. The PR domain
shares homology with the catalytic SET domain that characterizes a large group
of histone methyltransferases, and the zinc finger domains are known to be
involved in DNA binding and protein-protein interactions[3,6,7]. Members of the
PRDM family have been established as powerful transcriptional modulators, via
either direct histone methylation or by recruiting other regulatory modulators to
target promoters[1,3,8].

In primates, the PRDM family consists of 17 orthologous proteins, many of
which are present in different molecular forms by alternative splicing or by the
activation of alternative promoters[1,2,7-15]. This results in a highly diverse family
of transcriptional modulators that play a variety of essential roles in

development and physiology[3,6,7]. Unsurprisingly, the dysregulated activity of PRDM proteins has widespread effects that result in disease and developmental defects. PRDM1, also known as BLIMP1 (B-lymphocyte-induced maturation protein 1), has been identified as a master regulator of terminal differentiation in B cells. PRDM3 and PRDM16 also play key roles in hematopoiesis and stem cell homeostasis[1,8,11,13]. Constitutive knockout of several PRDM proteins, including PRDM1, 3 and 16, are either embryonic or neonatally lethal[11].

PRDM family members have also been associated with oncogenesis, whereas some other members have been identified as potential tumor suppressors. For instance, hypermethylation of the PRDM5 promoter has been frequently observed in gastric carcinogenesis, and loss of PRDM2 has been associated with the development of blast crisis in chronic myeloid leukemia[6,8,17]. Nonetheless, a number of PRDM proteins remain to be characterized, with little information available about either their expression profiles or their functional relevance. For example, PRDM7, is a primate-specific histone methyltransferase that has been associated with Li-Fraumeni syndrome and resulted from the duplication of the PRDM9 gene[17,18], which is the only hybrid sterility gene identified thus far in vertebrates. However, much still remains unknown regarding the roles of both proteins throughout development. The same occurs with the PRDM8 SET-domain, which is commonly located in the nucleus and is included as a specific HMTase (histone methyltransferase) proteins. Another members of the PRDM family are PRDM12, which has been

implicated in congenital pain insensitivity, and PRDM13, which is known to be expressed in the dorsal neural tube. While recent studies have shown that PRDM12 directs neuronal development towards the nociceptive neuron lineage, just a few of them have comprehensively assessed its expression throughout the course of embryonic development in vertebrates[15,19].

We recently developed two passive tissue clearing techniques (PACT), the process-separated PACT (psPACT) and the modified PACT (mPACT)[20,21], derived from the original CLARITY (Clear Lipid-exchanged Acrylamide hybridized Rigid imaging/immunostaining/*In situ* hybridization compatible Tissue hYdrogel) method[22] that allows for a three-dimensional microscopic analysis of intact whole tissue and organs. This technique has led to newfound understanding of cellular circuits in both physiological condition and disease, and allows for observation of subcellular structures, protein complexes, and biological networks with great spatial resolution. Compared to the original CLARITY method, psPACT and mPACT achieve tissue clearing with a greater transparency in a shorter timeframe. Nonetheless, it remains difficult to apply these techniques to more fragile tissues, such as those from developing mouse embryos. If possible, it could mean a great benefit to studies aiming to obtain three-dimensional information from these tissues.

Here, we present an optimized version of our PACT protocol that improves tissue rigidity, without the detriment to optical transparency, which was termed mPACT-A. We also described an mPACT-A protocol that is specially geared

towards handling small mouse embryos, which easily disintegrate when processed by conventional clearing technique. Using this technique, we investigated the expression of PRDM 7, 8, 12 and 13 throughout mouse embryonic development.

II. MATERIALS AND METHODS

1. Animals

Adult female BALB mice were purchased from Orient Inc. (Gyeonggi-do, Korea). Mouse embryos were isolated from E8.5 to E15.5 stages at the Cellular Reprogramming and Embryo Biotechnology Laboratory, in the Seoul National University's School of Dentistry. All procedures were carried out in strict accordance with the recommendations provided by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA) and were approved by Institutional Animal Care and Use Committee (IACUC) at Yonsei University.

2. Isolation of mouse tissue

Upon opening the mice thorax, an incision was made to the right atria of their hearts. Mice were then perfused with equal volumes of cold 0.1M phosphate-buffered saline (PBS) with 10unit/mL of heparine and 4% paraformaldehyde (PFA). Mouse embryos and central nervous system (CNS) tissue were then isolated using previously described methods.

3. Generation of the transparent embryos and CNS tissue

   A) E13.5 embryos and brain tissue clearing using optimized passive clearing methods

Cultures were established from fixed mouse embryos and brain tissues on a clean bench. The sample of four modified PACTs (psPACT, mPACT, psPACT-A and mPACT-A) was transferred to sufficient 4% PFA solution to cover the tissue in a 50mL tube, and then stored at 4°C for 24 hours. The fixed sample was washed for 1 hour with 0.1M PBS in a 50mL tube and then transferred to enough A4P0 solution (4% acrylamide in PBS) to cover the embry,o in a 50mL tube at 37°C for 24 hours. Next, the sample was covered with 0.25% VA-044 (Wako Pure Chemical Industries Ltd., Osaka, Japan) in 0.1M PBS in a 50mL tube at room temperature for 6 to 24 hours. The sample of mPACT-A was then transferred to A4P0. Then, the samples were embedded with nitrogen gas for 10min, and the tissue was transferred to a 50mL tube containing clearing solution (8% SDS in 0.1M PBS, pH 8.0) with or without 0.5% α-thioglycerol (Sigma-Aldrich, Inc., MO, USA) in amounts enough to cover the sample; to then get incubated while shaking at 150rpm at 37°C until the tissue cleared.

   B) Optical clearing of E7.5-11.5 embryos using mPACT-A

Embryos were fixed in 4% PFA at 4°C for 24 hours, and then washed with 0.1M PBS for 1 hour before being transferred to A4P0 at 37°C for 6 hours. Embryos were then covered in 0.25% VA-044 at room temperature for 6 hours, exposed

to nitrogen gas for 10 min, and re-transferred to A4P0 at 37℃ for 6 hours. Finally, embryos were incubated in clearing solution (8% SDS in 0.1M PBS, pH 8.0) with 0.5% α-thioglycerol at 37℃ in a shaking incubator at 150rpm until they achieved optical transparency. For more detailed instructions, see **Figure 2.**

4. Immunostaining

Cleared embryos were incubated in 0.1% Triton X-100 (Sigma-Aldrich, Inc., MO, USA) in 0.1M PBS for 2 hours, and blocked with 2% bovine serum albumin (BSA) in 0.1M PBS for 6 hours. Embryos were incubated with each PRDM 7, 8, 12 and 13 primary antibodies for 48 hours, followed by 3 washes in 0.1% Tween-20 in 0.1M PBS (PBST) for 2 hours each. Then, they were then incubated in secondary antibodies, lectin dye, and DAPI in 2% BSA for 48 hours. Then, nRIMS was prepared by mixing 0.8 g/mL of Nycodenz (Axis-shield Density Gradient Media, Oslo, Norway) in 30mL of base buffer (0.01% sodium azide and 0.1% Tween-20 in 0.1M PBS, pH 7.5). The labeled embryo was washed three times with PBST solution for 2 hours and stored in 5mL of nRIMS solution for 2 days. After adding the nRIMS solution to the confocal dish, small embryos of E7.5-11.5 were incubated in nRIMS there for 1-2 days, and covered using a coverslip with a diameter of 24mm. Detailed information about antibodies and dyes used in this study is provided in **Table 1.**

Table 1. Antibodies and dies information

	Antibody and dye	Marking target	Company	Cat No.	Ratio
1	PRDM7 rabbit antibody	Prdm7	Biorbyt	orb373884	1:100
2	PRDM9 rabbit antibody	Prdm8	Biorbyt	orb312589	1:100
3	PRDM12 rabbit antibody	Prdm12	Biorbyt	orb162475	1:100
	PRDM13 rabbit antibody	Prdm13	Biorbyt	orb312614	1:100
5	Goat anti-rabbit IgG H&L (Alexa Fluor® 488)	-	Abcam	ab150077	1:100
6	DyLight 594-labeled Tomato lectin	Blood vessel	Vector laboratories	DL-1177	-
7	DAPI Hoechst 33342	Nuclear	Invitrogen	R37605	-

5. Imaging processing

All clear images of embryos were captured using a digital camera (Canon 100D, Tokyo, Japan) and stereoscopic microscopes (SMZ745T; Nikon, Tokyo, Japan). Confocal microscopy was performed with an LSM-780 confocal microscope (Carl Zeiss, Oberkochen, Germany) at 10x magnification using the associated Zeiss software. Three dimensional images and videos were edited into serial image (Bitplane, Belfast, United Kingdom).

III. RESULTS

1. Generation of transparent mouse embryos using a modified passive clearing methods

To investigate the expression of PRDM 7, 8, 12 and 13 in intact embryos and CNS tissue, we sought to optimize our previously described psPACT and mPACT protocols to yield firmer tissue after clearing. The final steps of the

original psPACT and mPACT methods involve incubation of tissue in clearing solution consisting of just 8% (SDS) or 8% (SDS) with 0.5% α-thioglycerol, respectively. In this study, after embedding the tissue in 0.25% VA-044, we incubated tissues and embryos in A4P0 solution to improve the firmness of the resulting hydrogels, as shown in **Figure 1A.**

As shown in **Figure 1B and 1C**, the addition of the second A4P0 incubation in mPACT-A yielded firmer tissue after achieving optical clearance, as measured by the degree of resistance to gravity.
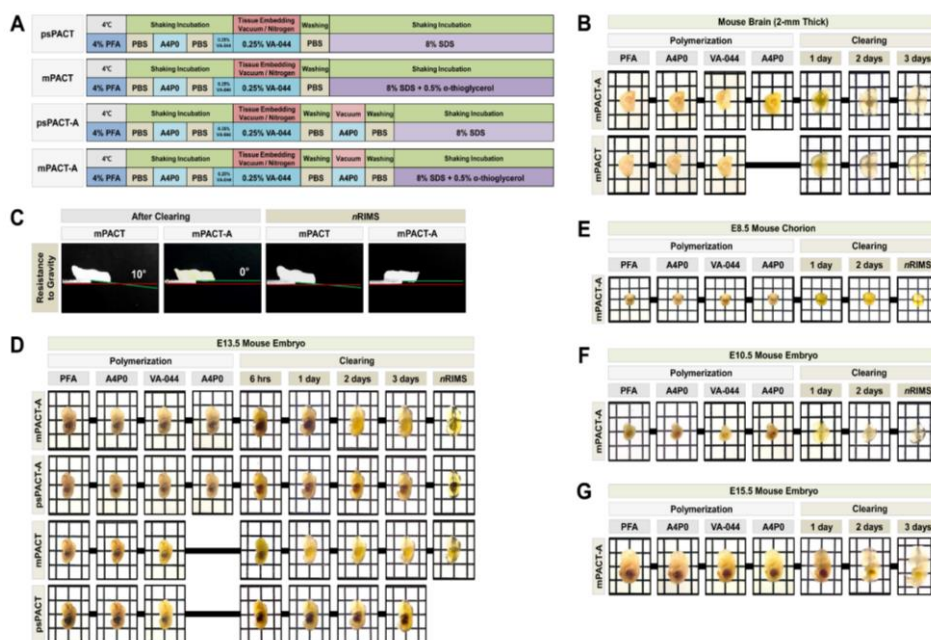

Figure1. Generation of transparent mouse embryos via psPACT and mPACT-A. (A) Scheme of psPACT, mPACT, psPACT-A and mPACT-A processes. (B) Brain 2mm clearing compare mPACT and mPACT-A. (C) Rigidity comparison between mPACT and mPACT-A. (D-G) Clearing of embryos at various developmental stages.

The improvement in tissue firmness was not at the expense of tissue clearance; as shown in **Figure 1D**, both psPACT-A and mPACT-A were able to achieve optical clearance of E13.5 mouse embryos within the same timeframe as the original protocols. Similar results were achieved for adult mouse brain, E8.5 mouse chorions, and mouse embryos at E10.5 and E15.5 **(Figure 1E-G).**

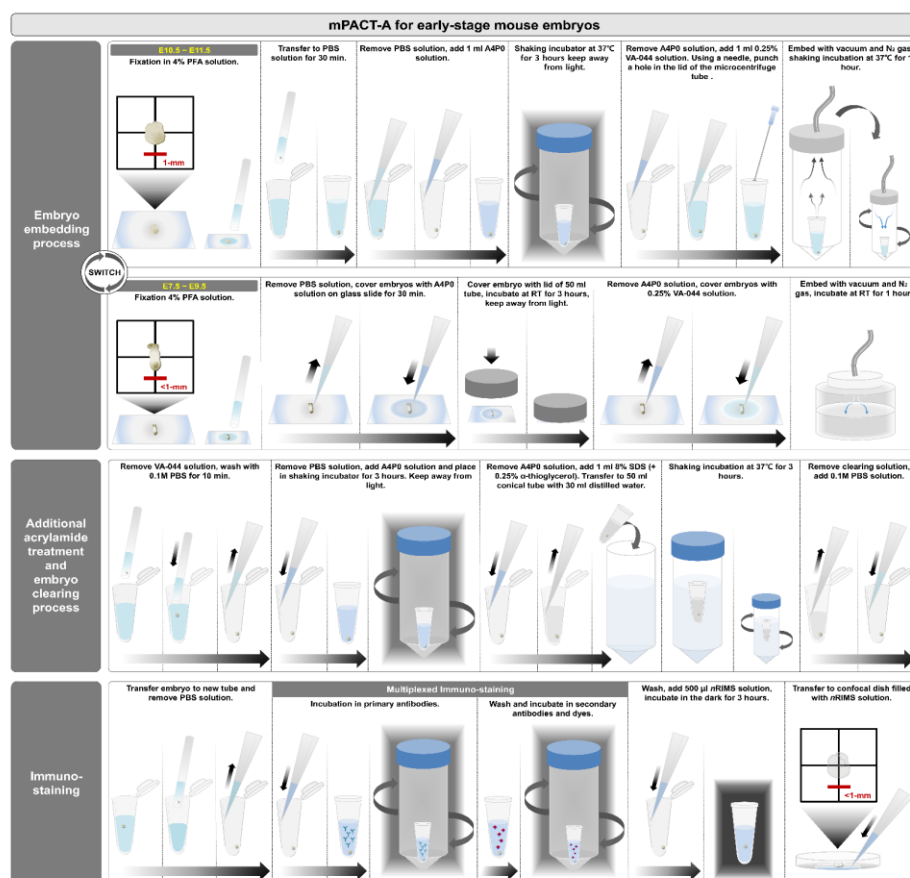**2.Development of embryo-specific mPACT-A to achieve tissue clarity in early stage embryos**

Figure 2. Scheme representing the optimized mPACT-A for mouse embryo.

In order to further optimize the mPACT-A method for mouse embryo during early development, we separate protocols for those between E7.5-E9.5 and E10.5-E11.5. In addition to the extra A4P0 incubation we added to the mPACT-A method, we added a second A4P0 incubation step after embryo embedding to improve tissue integrity.

We also optimized our protocol in such a way that it would be suitable for manipulating small-sized tissue, as early-stage mouse embryos are often less than 1mm in diameter. The workflow of this mPACT-A protocol is depicted in **Figure 2**.
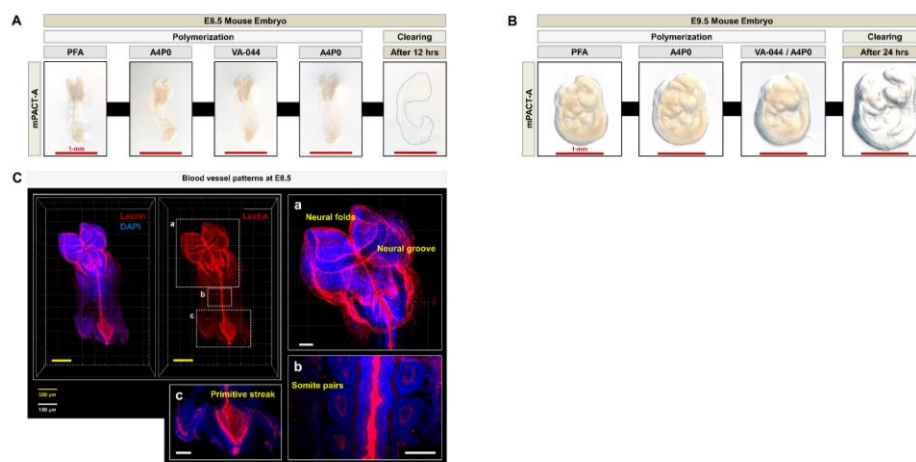

Figure 3. Stained blood vessels using the mPACT-A method. (A) Images of cleared E8.5 embryos. (B) Images of cleared E9.5 embryos. (C) Images of Immunostained blood vessels (red) and nucleus (blue).

As shown in **Figure 3A and 3B**, our embryo-specific mPACT-A protocol achieved tissue clearance in E8.5 and E9.5 mouse embryo after 12 and 24 hours,

respectively. Then, we tested the functional application of this technique by investigating early angiogenesis in E8.5 embryos with immunostaining for lectin during somitogenesis and notochord development. We were successfully able to generate z-stack images of blood vessel patterns throughout the entire mouse embryo at E8.5 (**Figure 3C**)**,** and observed their organization around the neural folds and neural groove, as well as the primitive streak and developing somites. This result provided a proof-of-concept demonstration that our embryo-specific mPACT-A method can be used to perform three-dimensional analysis of biological structures in whole intact embryos. Next, we chose four PRDM proteins: PRMD7, 8, 12 and 13, which were expected to be expressed in the CNS of the mouse embryo. As shown in **Figure 4,** PRDM proteins have different molecular structure by PR domain and zinc fingers.
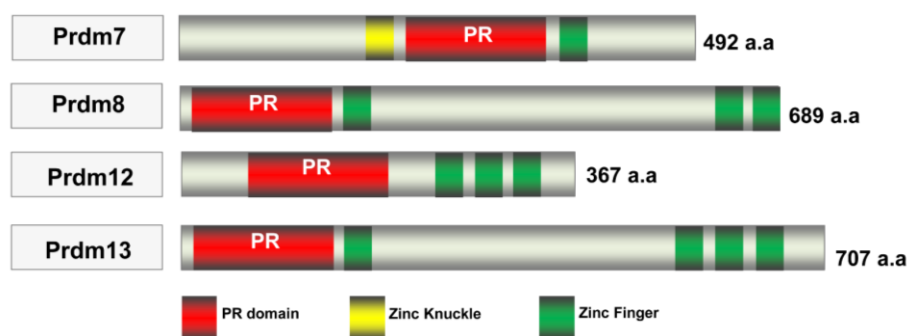

Figure 4. Scheme representing the PRDM molecular structure.

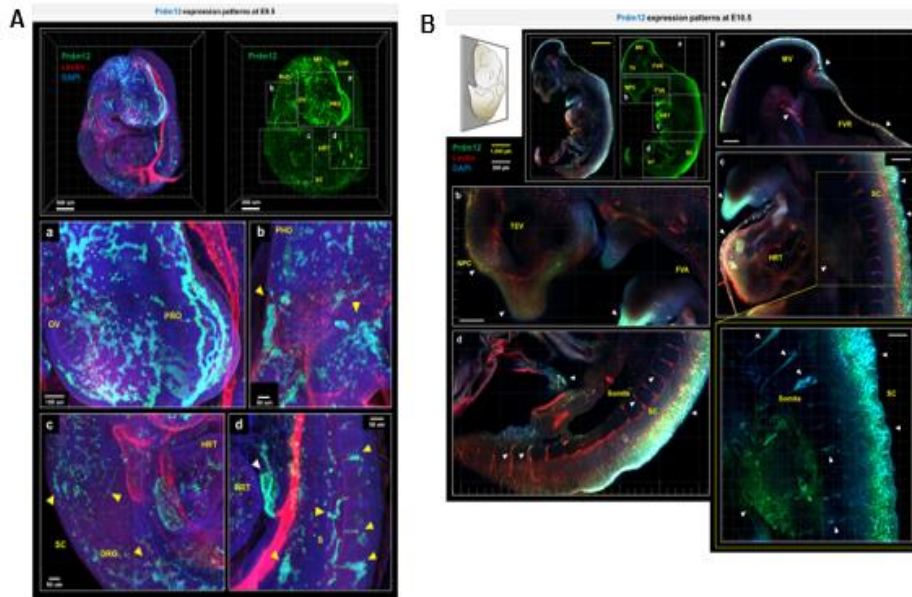### 3. PRDM12 expression in mouse embryos using mPACT-A

Figure 5. Profiling of PRDM12 expression during early mouse development in intact embryos via mPACT-A at E9.5 (A) and E10.5 (B).

PRDM family proteins function as either direct histone methyltransferase or modulators of epigenetic regulators, thus playing a variety of essential roles in physiology and development. Notably, PRDM12 has been implicated in congenital pain insensitivity, yet little is known about its expression profiles and biological functions in the developing vertebrate embryo. To investigate the expression pattern of PRDM12, we applied the mPACT-A technique to mouse embryos. At E9.5, PRDM12 was highly expressed in craniofacial structures and spinal cord, but its expression was also observed throughout the brain, especially in the optic vesicle, and the developing notochord (**Figure 5A**). At

E10.5, PRDM 12 expression continued to be observed in the developing CNS (**Figure 5B**). These results are in concordance with previous published studies on PRDM12 expression in mice.

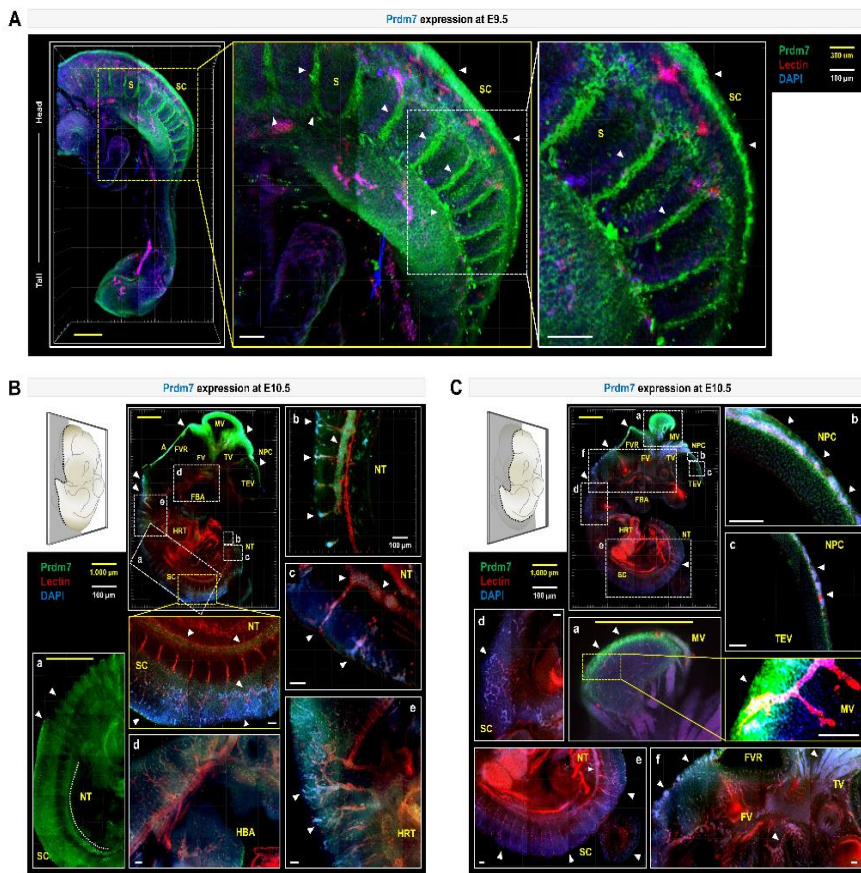**4. Expression patterns of PRDM 7 in mouse embryos at different stages**

Figure 6. Representative PRDM7 gene expression image in mouse embryo at E9.5 and E10.5. (A)At E9.5, PRDM7 expression is observed in somites and spinal cord. (B-C) Expression of PRDM7 at E10.5.

First, we investigated the body of an early mouse embryo (E9.5) to observe PRDM7 expression. We found that PRDM7 was expressed in spinal cord and somites (**Figure 6A**).

Because this study represents the first time that PRDM7 expression is described, we sought to further investigate its expression profile throughout different stages and regions of the developing mouse embryo. As shown in **Figure 6B and C**, we observed PRDM7 expression in different tissues at E10.5, including in those where it had been previously observed, somites and notochord, but also the developing brain and the adrenal medulla. Strong expression was observed in the spinal cord, with segmental expression in the spinal ganglia.
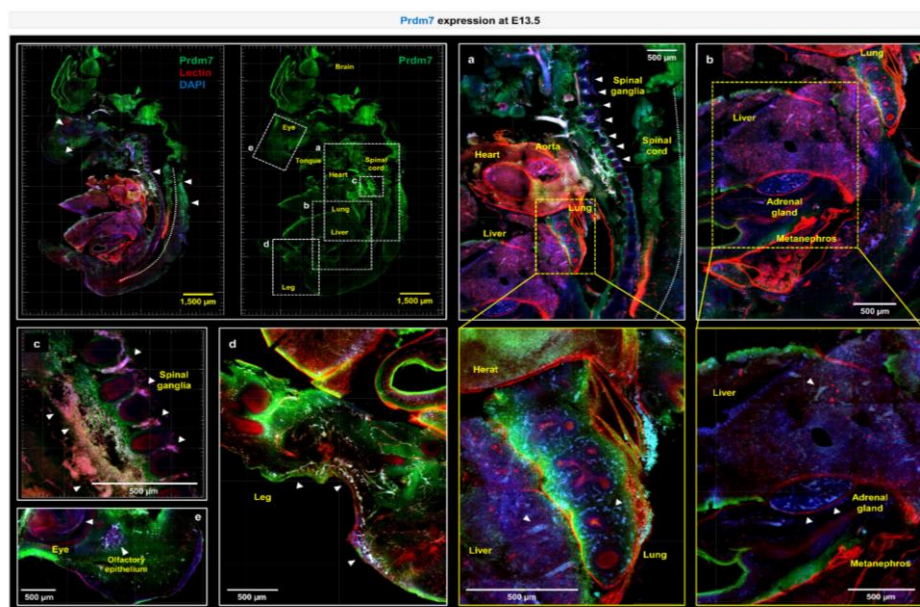

Figure 7. Representative PRDM7 gene expression image of sagittal section E13.5 embryonic whole body.

At E13.5, PRDM7 expression was still concentrated in the developing spinal cord and skeletal cartilage, but also observed in the lung, olfactory epithelium, adrenal medulla, the eye and the developing brain (**Figure 7**).

Since these PRDM7 expressing tissue were mainly derived from mesodermal or neural crest cell populations, the results suggest that PRDM7 is consistently expressed for mesodermal differentiation during mouse development.

5. Expression pattern of PRDM8 and PRDM13 in transparent mouse embryos

We also examined the expression pattern of PRDM8 and PRDM13 at E10.5 mouse embryos. As PRDM13 is known to be expressed in the dorsal neural tube, we expected to find PRDM13 also in the spinal cord, developing brain and tail region of mouse embryos. Our results showed that at E10.5, mPACT-A treated embryos had the PRDM13 expression concentrated in spinal cord, somites, neural tube and developing brain regions (**Figure 8A**).

In **Figure 8B**, the expression pattern of PRDM8 shows this protein is produced in the dorsal neural tube, somites, spinal cord and developing brain regions at E10.5. Strong expression was observed in notochord, adrenal medulla and also in brain regions.

17
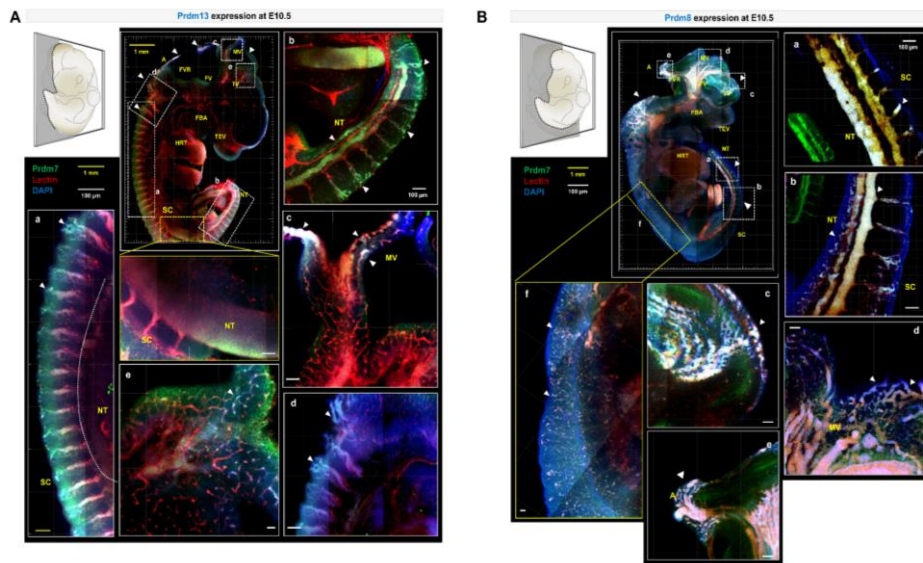
Figure 8. Representative PRDM13 and PRDM8 gene expression at E10.5 stage. (A) PRDM13 expression in E10.5 (B) RPDM8 expression in E10.5

IV. DISCUSSION

After applying the mPACT-A to mouse embryos, we demonstrated its viability by investigating the expression of four PRDM family proteins in mouse embryos, PRDM 7, 8, 12, and 13, which have been implicated in congenital disorders, but nonetheless are understudied compared with other PRDM proteins.

As determined by a previous study, PRDM12 is known play a role in the development of neurons. Accordingly, we show that PRDM12 is expressed in the developing nervous system both at E9.5 and E10.5 including the notochord, dorsal root ganglia and spinal cord as well as other regions in the developing

18

brain.

We also observed PRDM7 expression primarily in craniofacial structures and neural structures such as notochord and the olfactory epithelium, with an especially high expression in the spinal ganglia. At E9.5, PRDM7 was expressed in the somites formation. At E10.5, PRDM7 was expressed in brain epithelium, spinal cord and concentrated in the neural tube. At E13.5, PRDM7 expression was also observed in the lung, adrenal medulla and the eye. Comparing the pattern between each development stage, PRDM7 was concentrated in somite pairs and spinal cord at early stages, to then spread to developing brain regions and the CNS. Later, PRDM7 spread to the whole body, but kept concentrated in the spinal cord, somite pairs and brain regions which derived from the mesoderm. Notably, there are no previously published studies reporting tissue-specific PRDM7 expression, thus our study represents the first time in which PRDM7 expression is profiled.

PRDM13 was examined at E10.5, and was found to be expressed in spinal cord, neural tube, somite pairs and developing brain epithelium. At the same stage, PRDM8 expression was found in the spinal cord, neural tube and developing brain regions.

In a previous study, results of PRDM family expression patterns using *in situ* hybridization (ISH) assay, PRDM8, 12 and 13 were found to be highly expressed in the spinal cord region. Similarly, our results by using mPACT-A technique shows that PRDM8, 12 and 13 expression in spinal cord is as

concentrated, but also in neural tube and brain regions. Moreover, there were no previous studies about the expression pattern of PRDM7 in vertebrate development. We found it shows similar patterns in spinal cord, neural tube and developing brain regions in various development stages. Comparing with previous imaging studies, our mPACT-A technique could enable us to see specific regions such as the neural tube and somites, as well as the level of expression with high-resolution imaging. This level of resolution of expression level is possible since our mPACT-A technique clears the tissue while keeping it intact. As for PRDM12, it is known to have a role in the development of sensory neurons and it is expressed in the CNS. The high levels of PRDM expressions observed in the developing mouse nervous system suggest potential roles in neural development, which is represented in our result by using passive tissue clearing technique and high-resolution imaging analysis: PRDM7, 8, 12 and 13 are expressed in the mesoderm and developing CNS.

V. CONCLUSIONS

The development of a new tissue clearing technique allows the analysis of biological structures using three-dimensional intact organs. When used to study developing embryos, the mPACT-A technique showed greater rigidity after clearing. Also, a shorter time was required to apply it compared to PACT, obtaining the same degree of transparency. We demonstrated the feasibility of this modified technique by investigating the expression of PRDM 7, 8, 12 and 13 which plays critical roles in vertebrate development, showing their expression in mesodermal regions, spinal cord, neural tube and developing brain regions as similar in each other. Future functional studies are needed to further characterize the biological role of PRDM7, 8, 12 and 13, whose role remains elusive beyond the regulation of disease which occur during neural development.

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ABSTRACT(IN KOREAN)

Expression Pattern of PRDM Family Using Tissue Clearing Technique in Mouse Micro-embryo

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강 혜 원

PRDM 단백질군은 다양한 질병에서 종양억제제로서의 잠재적인 역할을 한다고 알려져 있다. PRDM의 발현 양상을 고해상도로 분석하는 것은 훗날 연구의 질병의 바이오 마커로써 사용 될 수 있는 PRDM의 발현 위치를 확인 할 수 있다.

CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/*In situ* hybridization-compatible Tissue hYdrogel) 과 같은 조직 투명화 방법의 개발은, 온전한 조직 전체에서 생물학적 구조를 3차원적인 분석을 가능하게 하여 그들의 관계와 생물학적 회로를 더 많이 이해할 수 있도록 합니다. 그럼에도 불구하고, 연구에 따르면, 구조적인 안정성을 유지하며, 조직의 붕괴를 방지하여 발생 중의 배아와 같은 연약한 조직에 이러한 기술의 적용을 할 수 없는 문제가 보고되었다.

본 연구는 투명화된 조직의 견고함을 향상시키고, 수동형 조직 투명화 기술인 mPACT-A를 제시한다. mPACT-A 기술은 작고 연약한 쥐 배아를 다루기에 특화하였다.

본 연구는 다양한 쥐의 배발생단계에 따른 투명화된 쥐 배아에서 PRDM 단백질군 중 PRDM7, -8, -12, -13 단백질들의 발현 양상을 비교적으로 이해하기 위한 조사를 하였다.

본 연구에서는 다양한 쥐 배아 발생 단계의 쥐 배아에서 신경계 발생 과정에서 PRDM7, -8, -12, -13이 강하게 발현하는 것을 확인하였다. 이러한 결과는 추가적인 기능적 연구들에 의하여 신경발생에 중요한 역할이 제시 될 것 이다.

핵심되는 말 : PRDM, 배아, 조직투명화 기술, mPACT, psPACT